Salerno, J. S. (1984) *J. Biol. Chem. 259*, 2331-2336.
Santos, H., Moura, J. J. G., Moura, I., LeGall, J., & Xavier, A. V. (1984) *Eur. J. Biochem. 141*, 283-296.
Seibert, M., & DeVault, D. (1970) *Biochim. Biophys. Acta 205*, 200-231.
Senn, H., Keller, R. M., & Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun. 92*, 1362-1369.
Shill, D. A., & Wood, P. M. (1984) *Biochim. Biophys. Acta 764*, 1-7.
Shopes, R. J., Levine, L. M. A., Holten, D., & Wraight, C. A. (1987) *Photosynth. Res. 12*, 165-180.
Skulachev, V. P., Drachev, L. A., Dracheva, S. M., Konstantinov, A. A., Semenov, A. Y., & Shuvalov, V. A. (1987) in *Cytochrome Systems* (Papa, S., Chance, B., & Ernster, L., Eds.) pp 609-616, Plenum Press, New York and London.
Taylor, C. P. S. (1977) *Biochim. Biophys. Acta 491*, 137-149.
Tiede, D. M., Leigh, J. S., & Dutton, P. L. (1978) *Biochim. Biophys. Acta 503*, 524-544.
Weyer, K. A., Lottspeich, F., Gruenberg, H., Lang, F., Oesterheld, D., & Michel, H. (1987) *EMBO J. 6*, 2197-2202.
Widger, W. R., Cramer, W. A., Herrmann, R. G., & Trebst, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 674-678.

Synthesis, Location, and Lateral Mobility of Fluorescently Labeled Ubiquinone 10 in Mitochondrial and Artificial Membranes†

K. Rajarathnam,‡ J. Hochman,§ M. Schindler, and S. Ferguson-Miller*
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824
Received September 13, 1988; Revised Manuscript Received December 20, 1988

ABSTRACT: To explore the influence of the long isoprene chain of ubiquinone 10 (UQ) on the mobility of the molecule in a phospholipid bilayer, we have synthesized a fluorescent derivative of the head-group moiety of UQ and measured its lateral diffusion in inner membranes of giant mitochondria and in large unilamellar vesicles. The diffusion coefficients, determined by the technique of fluorescence redistribution after photobleaching, were 3.1×10^{-9} cm^2 s^{-1} in mitochondria and 1.1×10^{-8} cm^2 s^{-1} in vesicles. Similar diffusion rates were observed for fluorescently labeled phosphatidylethanolamine (PE) with the same moiety attached to its head group (4-nitro-2,1,3-benzooxadiazole: NBD). Fluorescence emission studies carried out in organic solvents of different dielectric constants, and in vesicles and mitochondrial membranes, indicate that NBDUQ is located in a more hydrophobic environment than NBDPE or the starting material IANBD (4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitro-2,1,3-benzoxadiazole). Fluorescence quenching studies carried out with $CuSO_4$, a water-soluble quenching agent, also indicate that NBDUQ is located deeper in the membrane than NBDPE. These results suggest that ubiquinone and PE are oriented differently in a membrane, even though their diffusion rates are similar. Conclusions regarding whether or not diffusion of UQ is a rate-limiting step in electron transfer must await a more detailed knowledge of the structural organization and properties of the electron transfer components.

Ubiquinone 10 (UQ)[1] is an integral component of the electron transfer chain that mediates the transfer of electrons between respiratory complexes in the inner mitochondrial membrane and participates in the translocation of protons across the membrane. A hydrophobic molecule with a long, somewhat rigid, isoprenoid side chain of 50 Å, UQ in an extended form would span the bilayer (Trumpower, 1981). It was originally proposed by Green (1962) that UQ could act as a mobile electron carrier, because of its structure and presence in excess over its redox partners (about 20-fold molar excess over cytochrome bc_1; Capaldi, 1982). It has become increasingly evident that diffusion of UQ is important for electron transfer, but whether its diffusion is rate limiting remains to be established. To gain insight into this question, it is important to know the diffusion rates of UQ, the activation energy for the diffusion process compared to that of the electron transfer process, the K_m values for the interaction of native quinone with the electron transfer complexes in the mitochondrial membrane, and the average distance UQ has to diffuse to accomplish electron transfer. Currently, there is controversy regarding all these values.

Gupte et al. (1984) have measured the diffusion of a fluorescent derivative of a ubiquinone analogue which has an alkyl side chain corresponding in length to only two isoprene units. The lateral diffusion coefficients they determined by fluorescence redistribution after photobleaching (FRAP) were 3×10^{-9} cm^2 s^{-1} in the mitochondrial membranes and 5.5×10^{-8} cm^2 s^{-1} in phospholipid vesicles (Hackenbrock et al., 1986a). From diffusion measurements as a function of temperature (Hackenbrock et al., 1986b) and as a function of

† This work was supported by NIH Grant GM26916 (S.F.-M.).
* To whom correspondence should be addressed.
‡ Present address: Department of Chemistry, University of California Davis, Davis, CA 95616.
§ Present address: INTERx Research Corp., 2201 W. 21st St., Lawrence, KS 66046.

[1] Abbreviations: BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; UQ, coenzyme Q_{10}, ubiquinone 10; DMAP, (dimethylamino)pyridine; IANBD, 4-[*N*-[(iodoacetoxy)ethyl]-*N*-methylamino]-7-nitro-2,1,3-benzoxadiazole; NBDUQ, 4-[*N*-(acetoxyethyl)-*N*-methylamino]-7-nitro-2,1,3-benzoxadiazole–ubiquinone 10; NBDPE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoylphosphatidylethanolamine; THF, tetrahydrofuran; Thiolyte, monobromobimane; TLC, thin-layer chromatography.

0006-2960/89/0428-3168$01.50/0

increased membrane dilution with phospholipids (Schneider et al., 1982), they propose that diffusion of UQ is rate limiting in electron transfer.

In contrast, Fato et al. (1986) have measured the diffusion of UQ in small sonicated vesicles and submitochondrial particles by the technique of collisional quenching using fluorescently labeled fatty acids. They report diffusion coefficients of 1×10^{-6} to 2×10^{-7} cm^2 s^{-1}. They have calculated the activation energy for diffusion [a value different from that obtained by Hackenbrock et al. (1986b)] by measuring quenching constants as a function of temperature, while obtaining the K_m values for UQ with cytochrome bc_1 and NADH dehydrogenase from kinetic measurements. They conclude from these studies that diffusion of UQ is not rate limiting in electron transfer.

A major discrepancy in the data of these investigators that affects their conclusions is the difference of 2 orders in magnitude in the apparent diffusion coefficients for UQ. Two possible reasons for this discrepancy can be considered. First, there could be systematic errors in the techniques or calculations involved in obtaining the coefficients; i.e., FRAP could be underestimating, or collision quenching overestimating, the mobility (or both). FRAP is capable of measuring diffusion values of the order of 10^{-7}–10^{-8} cm^2 s^{-1} (Fahey & Webb, 1978), and hence, the diffusion rates of 10^{-8}–10^{-9} cm^2 s^{-1} reported by Hackenbrock and co-workers are unlikely to be limited by the technique. On the other hand, Fato et al. (1986) measure the bimolecular collision constants and calculate the diffusion coefficients by making use of the modified Smoluchowski's equation (Lakowitz & Hogen, 1980) which assumes that the quencher and the quenched molecule are spherical and tumbling isotropically in the membrane. However, the diffusants, UQ and anthroyloxy fatty acids, are not spherical, the orientation is unlikely to be isotropic, and the membrane diffusion is likely to be two-dimensional rather than three-dimensional. In fact, it has been shown that the motion of the anthracene ring of *n*-(anthroyloxy) fatty acids when incorporated into phospholipids is hindered and rotational constraints would make its motion highly anisotropic (Badley et al., 1973). Thus, the possibility of a systematic error in the calculations of diffusion coefficients from the collisional quenching data must be considered (see Discussion).

A second reason for the difference in the diffusion values could be related to the use of a short-chain ubiquinone analogue by Hackenbrock's group in their studies. It is well established that short-chain quinones orient themselves parallel to a phospholipid in a bilayer with the quinone moiety lying near the membrane surface (Katsikas & Quinn, 1983). This fact would account for the diffusion rate of the ubiquinone analogue being similar to that of a phospholipid. In contrast, long-chain quinones appear to be located deeper in the membrane, possibly with the isoprenoid chain lying in the midplane of the bilayer (Crane, 1977; Ulrich et al., 1985; Michaelis & Moore, 1985; Ondarroa & Quinn, 1986; Cornell et al., 1987). It has been postulated that UQ could diffuse very fast (10^{-6} cm^2 s^{-1}) if it were located between the bilayer leaflets (Millner & Barber, 1984). Therefore, the possibility must be considered that the diffusion of the ubiquinone analogue may not represent that of UQ.

To test the importance of the isoprenoid chain in determining the location and mobility of UQ in membranes, we have synthesized the first-reported fluorescent derivative of ubiquinone 10 and have measured its diffusion in mitochondrial membranes and phospholipid vesicles. We also report experiments designed to locate the position of the fluorescently labeled ubiquinone by fluorescence emission and fluorescence quenching studies. Our results show that the 4-nitro-2,1,3-benzoxadiazole (NBD) moiety attached to the head group of UQ is located deeper in the membrane than the same fluorescent moiety attached to the head group of a phospholipid. Nevertheless, the diffusion rates of the two derivatized species are similar.

Experimental Procedures

Materials. The following materials were obtained from the sources indicated: cuprizone, Aldrich Chemical Co., recrystallized from 95% ethanol according to Bowman and Tedeschi (1983); asolectin, Associated Concentrates, Long Island, NY; NBD-labeled phospholipids, Avanti, Birmingham, AL; IAN-BD, Molecular Probes, Eugene, OR; preparative thin-layer chromatography (TLC) plates, Analtech, Newark, DE; (dimethylamino)pyridine (DMAP), recrystallized from dichloromethane, ubiquinone 10, and CCCP, Sigma Chemical Co.

Preparation of Giant Mitochondria. Fifteen to seventeen day old mice (Swiss Albino ICR) are fed a diet containing 3 g of cuprizone in 500 g of rodent chow. Giant mitochondria from these mice are prepared 6–12 days after initiation of the diet, essentially according to the procedure of Hochman et al. (1985). Livers from three to four mice are minced in 5 mL of isolation buffer [220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.05% BSA, pH 7.4 (with Tris)], washed a few times in the same buffer, and homogenized twice in a loose-fitting glass Dounce homogenizer. The homogenate is centrifuged at 120*g* for 1 min, the supernatant is saved, and the pellet is homogenized with the same amount of buffer and centrifuged again at 120*g* for 1 min. The supernatants are pooled and layered on 10 mL of 0.5 M sucrose and centrifuged at 730*g* for 5 min in a swinging-bucket bench-top centrifuge. The top layer containing smaller mitochondria is discarded along with a loose bloody pellet at the bottom of the tube. The rest of the 0.5 M sucrose layer and the interface are gently resuspended. The solution is diluted to 0.3 M sucrose with cold distilled water and then spun at 750*g* for 5 min. The pellet containing the giant mitochondria is suspended in 5 mL of isolation buffer, layered on 0.5 M sucrose, and centrifuged at 240*g* for 3 min in a bench-top centrifuge, and the top layer excluding the interface is collected and pelleted at 750*g* for 5 min. The giant mitochondria are depleted of outer membrane by first swelling the mitochondria in 5 mL of 5 mM Tris–phosphate (pH 7.5) for 5 min and then adding an equal volume of shrinking buffer (1.8 M sucrose, 2 mM ATP, 2 mM Mg^{2+}) and incubating for another 5 min. The mixture is then sonicated for 20 s (setting 3, Model W-225, Heat Systems Ultrasonics, Inc.), and the mitoplasts are pelleted at 1900*g* for 10 min. The mitoplasts are suspended in 0.1–0.2 mL of 250 mM mannitol/50 mM Hepes (pH 7.2) buffer, and the amount of protein is determined by a modified Lowry assay using bicinchoninic acid (BCA) (Smith et al., 1985).

Preparation of Cell-Size Vesicles for FRAP. Unilamellar phospholipid vesicles are prepared essentially according to the procedure of Mueller et al. (1983). Asolectin (6.25 mg), α-tocopherol (0.04 mg), and NBDUQ (0.125 mg) are dissolved in chloroform in a test tube to give a 1:50 (w/w) ratio of NBDUQ to phospholipid. This is made to a final volume of 2 mL in chloroform/methanol/0.5 M NaCl (2:2:1.8 v/v) in order to make it biphasic. The mixture is vortexed and then spun at room temperature for 5 min in a bench-top centrifuge. The upper aqueous layer is discarded, and the chloroform layer is filtered through anhydrous $MgSO_4$ into a 25-mL Erlenmeyer flask. The filtrate is dried under argon, and 25 mL of 0.05

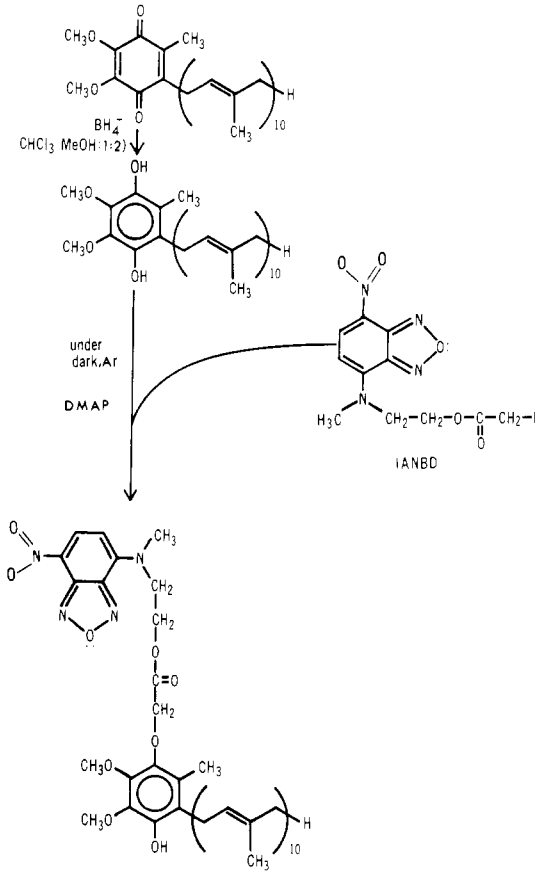

FIGURE 1: Reaction scheme for synthesis of NBDUQ. Ubiquinone is reduced with borohydride (BH_4^-) in chloroform/methanol (1:2 v/v). The reaction with IANBD is carried out in tetrahydrofuran in the dark, under argon (Ar), with DMAP as a catalyst. Further details are given under Experimental Procedures.

mM NaCl solution is added gently into the flask. The flask is wrapped with silver foil and is kept at 4 °C. Vesicles form at the bottom of the flask after 1–2 days and are stable over a period of 1–2 weeks.

Synthesis of NBDUQ. The reaction scheme for synthesis of NBDUQ is given in Figure 1. Ubiquinone (5 mg) is dissolved in 1 mL of chloroform/methanol (1:2 v/v) in a screw-top culture test tube and reduced with a few grains of sodium borohydride. The solution turns colorless, and there is a brisk effervescence of hydrogen gas. The solution is stirred until the evolution of hydrogen ceases and then dried with argon. A total of 1.7 mg of (dimethylamino)pyridine (DMAP) (3:4 mol/mol) and 4.7 mg of IANBD (2:1 mol/mol) are added to the reduced ubiquinone, the mixture is suspended in 1 mL of dry tetrahydrofuran (THF) and flushed with argon, and the reaction is carried out overnight at room temperature. The reaction mixture is dried with argon and dissolved in 300 μL of chloroform/methanol (2:1) and applied to a preparative TLC (Uniplate-T, Analtech). The plate is chromatographed in chloroform/hexane/methanol (100:50:2.5 v/v) for 15 min. NBDUQ (R_f = 0.55) runs between IANBD (R_f = 0.4) and ubiquinone (R_f = 0.85) and is scrapped from the plate and extracted with chloroform/methanol (2:1). The product is filtered, dried with argon, and stored at −20 °C until use.

FRAP Experiments. Glass slides are washed and rinsed with distilled water and ethanol and then allowed to air-dry. For experiments using liposomes, the clean slides are coated with gelatin according to Bowman and Tedeschi (1983). Labeled mitoplasts or liposomes (15–20 μL) are applied to a slide, and the cover slips are sealed around the edges with paraffin to prevent evaporation. Diffusion measurements were

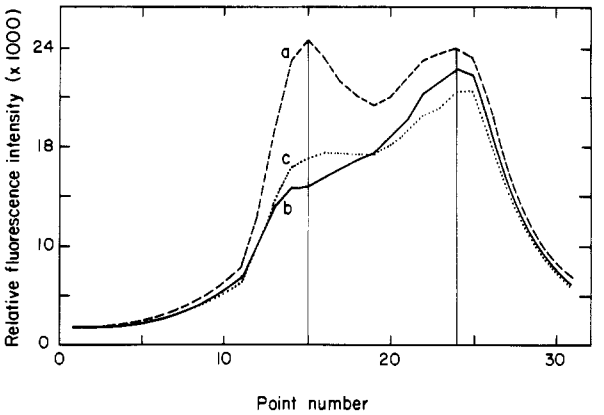

FIGURE 2: Representative FRAP experiment showing fluorescence intensity scans of a NBDUQ-labeled large phospholipid vesicle: (a) before bleaching (t < 0 s); (b) after bleaching (t = 0 s); (c) after bleaching (t = 5 s). The diameter of the vesicle (distance between fluorescence maxima) was 8.7 μm.

performed by the technique of fluorescence redistribution after photobleaching using the instrumentation and analysis developed by Koppel and colleagues (Koppel, 1979; Koppel et al., 1980). The low-intensity laser beam is scanned across the fluorescently labeled membrane, and profiles of the fluorescence emission are recorded. A sample profile for a phospholipid vesicle labeled with NBDUQ is shown in Figure 2a. The peaks indicate that the fluorescent probe is associated with the membrane, giving a more intense fluorescence at the edges. A higher intensity pulse is applied to one edge of the vesicle resulting in bleaching (chemical destruction) of the probe in this region and giving the profile seen in Figure 2b. The decay of the photobleach-induced fluorescence intensity gradient of the fluorescent probe (Figure 2c) is followed with sequential scans, and the data are analyzed by a normal-mode analysis for diffusion on spherical surfaces as described by Koppel et al. (1980). NBD fluorescence is monitored with an incident wavelength of 477 nm and a combination of a Leitz dichroic mirror TK 510 and a barrier filter K 530.

Incorporation of Fluorescent Probe into Mitoplasts. To 100 μL of mitoplasts (~10 mg of protein/mL) in 250 mM mannitol/50 mM Hepes buffer (pH 7.2), 3–5 μL of NBDUQ (3–5 mg/mL) or 3 μL of head-group NBDPE (1 mg/mL) in ethanol and 5 μL of BSA (1 mg/mL) are added and incubated for 15 min at room temperature. The mitoplasts are then swollen with 3 volumes of water over a period of 10 min on ice. These conditions, especially the presence of BSA, were important for achieving sufficient incorporation to perform the FRAP experiments. To quantitate the amount of NBDUQ incorporated into the mitochondrial membranes under the conditions used for FRAP, 50 μL of NBDUQ-labeled mitoplasts (3 mg of protein/mL) was diluted to 1 mL with 62.5 mM mannitol/12.5 mM Hepes (pH 7.2) buffer and centrifuged at 4000g for 10 min. Unincorporated NBDUQ was extracted from the supernatant by partitioning it into an organic phase by adding chloroform, methanol, and 0.5 M NaCl to a ratio of 2:2:1.8 (v/v) and spinning in a bench-top centrifuge for 5 min. The upper aqueous layer was discarded, and the chloroform layer was filtered through anhydrous $MgSO_4$ and dried with argon. The pelleted mitochondria were resuspended in 0.1 mL of the mannitol/Hepes buffer mentioned above, and the incorporated NBDUQ was extracted by addition of chloroform, methanol, and 0.5 M NaCl to a final volume of 1:2:0.8 (v/v). The mixture was spun as before, and the supernatant was transferred to another test tube and made biphasic by addition of chloroform, methanol, and 0.5

M NaCl to a final ratio of 2:2:1.8 (v/v). The extraction was carried out as described for the supernatant. From the absorbance at 457 nm of the mitochondrial and supernatant extracts redissolved in chloroform, the amount of NBDUQ incorporated into the membrane was calculated. In the case of the liposomes, the final concentration of NBDUQ or NBDPE is determined from the amount originally added to the phospholipid mixture.

Fluorescence Emission and Quenching Measurements. Emission maxima of the fluorescent probes in organic solvents and lipid membranes were determined at an excitation of 468 nm with a Perkin-Elmer 550-60 spectrofluorometer. Quenching experiments with $CuSO_4$ were carried out as follows. To a cuvette containing a 1-mL solution of mitochondria labeled with NBDUQ or NBDPE, $CuSO_4$ (3 mM) was added in 5-μL aliquots. For the vesicles, 3- or 5-μL aliquots of a 1 mM solution of $CuSO_4$ were added. After each addition, the sample was mixed and then allowed to equilibrate before measurement of the fluorescence intensity. The NBD moiety was excited at 468 nm, and the emission was monitored at 527 nm for NBDUQ and 536 nm for NBDPE. Slit width was 5 nm for the excitation beam and 10–13 nm for the emission beam.

Effect of NBDUQ on Succinate Oxidase Activity. Succinate oxidase activity of mitochondrial inner membranes, with and without labeling with NBDUQ or NBDPE, was measured on a Gilson polarograph in 1.75 mL of 250 mM mannitol, 50 mM Hepes, (pH 7.2), 13.8 mM succinate, 7.6 μM cytochrome *c*, and 0.55 μM CCCP. Turnover numbers (TN) were calculated from the rates of O_2 consumption multiplied by 4 to give the nanomoles of cytochrome *c* required to reduce 1 nmol of O_2 and divided by the total nanomoles of cytochrome aa_3 present in the reaction vessel.

Results

Synthesis and Characterization of NBDUQ. We are reporting for the first time the synthesis of a fluorescent derivative of ubiquinone 10, NBDUQ. Reduced ubiquinone was reacted with IANBD using DMAP as a catalyst in THF to obtain NBDUQ (yield = 5%). NBDUQ was purified in a single step by preparative TLC as described under Experimental Procedures. Good separation between the starting materials (IANBD, UQ) and NBDUQ is achieved, and when NBDUQ is rerun on the preparative TLC, the product runs as a single spot. There are two hydroxyl groups on the quinol, both of which react to give two isomer forms that can be separated by running the TLC for a longer time. A similar procedure was used to synthesize a Thiolyte (monobromobimane, Calbiochem Co.) derivative of ubiquinol, illustrating the general applicability of the method for modifying the hydroxyl groups of the quinol.

NBDUQ is stable when kept at −20 °C in dried form. In solution (ethanol) there is some breakdown, 5–10% in 24 h. Normally, NBDUQ was freshly synthesized as necessary and, once dissolved in ethanol, was used within 6 h. Any contribution of small amounts of hydrolyzed NBD probe to the mobility or quenching measurements would be negligible as it is more water soluble, partitions less into the membranes, and has an emission maximum at 540 nm rather than 527 nm for NBDUQ.

Figure 3 shows the absorption characteristics of NBDUQ (A) and both the starting materials, IANBD (B) and reduced ubiquinone (C). NBDUQ has a UV–visible spectrum similar to that of the sum of the reactants and an extinction coefficient of 12 mM^{-1} cm^{-1} at 470 nm. NMR spectra of NBDUQ show chemical shift values characteristic of both UQ and IANBD

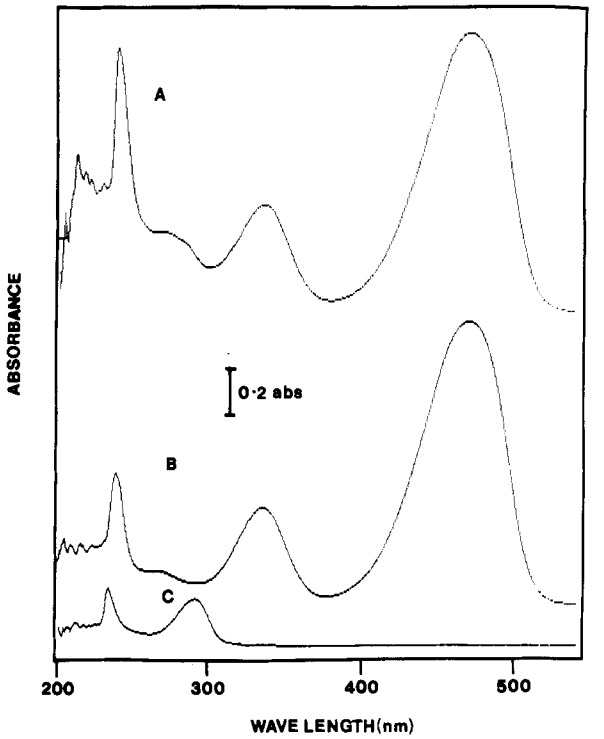

FIGURE 3: Absorption spectra of NBDUQ (A), IANBD (B), and ubiquinone (C) measured in chloroform at concentrations of 56, 36, and 36 nmol/mL, respectively.

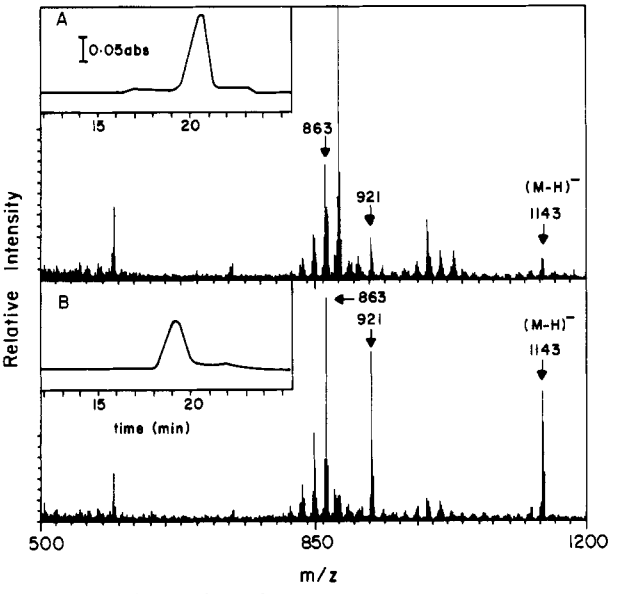

FIGURE 4: Elution profiles of two isomers of NBDUQ from HPLC and their mass spectra. The two isomers of NBDUQ were separated by preparative TLC under identical conditions as used during purification (Experimental Procedures) except that the chromatography time was increased to 50 min. HPLC of the purified products was performed on a Waters C-18 reverse-phase column (3.9 mm × 30 cm). The solvent system was methanol with 0.7% $NaClO_4 \cdot H_2O$ and 0.1% (v/v) 70% $HClO_4$ (Katayama et al., 1980). The flow rate was 1 mL/min, and elution was monitored at 254 nm. Fast atom bombardment (FAB) mass spectra were obtained on a JEOL HX-110 HF mass spectrometer, operating in the negative mode. The molecular ion is indicated by $(M - H)^-$. The FAB matrix used was triethanolamine.

(Rajarathnam, 1987). The two isomers of NBDUQ were separated by TLC and characterized by HPLC on a reverse-phase column as shown in Figure 4. They had similar absorption spectra (not shown), and their isomeric character was unequivocally confirmed by mass spectrometry (Figure

Table I: Lateral Diffusion of NBDUQ and NBDPE in Mitochondria and Phospholipid Vesicles[a]

probe	diffusion coeff (cm^2 s^{-1})	
	mitochondria[b]	vesicles[c]
NBDUQ	$(3.1 \pm 1.0) \times 10^{-9}$ ($n = 4$)	$(1.1 \pm 0.2) \times 10^{-8}$ ($n = 6$)
NBDPE	$(3.0 \pm 0.4) \times 10^{-9}$ ($n = 4$)	$(1.3 \pm 0.3) \times 10^{-8}$ ($n = 3$)

[a] Diffusion coefficients were measured as described in the legend to Figure 5. [b] Mitochondria were labeled with fluorescent molecules as described in the legend to Figure 5. [c] Vesicles containing fluorescent probes were prepared essentially according to Mueller et al. (1983) as given under Experimental Procedures.

4). Both the spectra have peaks corresponding to the molecular weight of NBDUQ, and they have similar fragmentation patterns. Some of the prominent peaks are $[NBDUQ - H]^-$ = 1143 (molecular ion), $[UQOCH_2COO - H]^-$ = 921, $[UQ - H]^-$ = 863. Generally, the mixture of both isomers of NBDUQ was used for the experiments.

Preparation and Characterization of Giant Mitochondria and Phospholipid Vesicles. Lateral diffusion coefficients of NBDUQ and NBDPE were measured in inner membranes of giant mitochondria prepared from cuprizone-fed mice and in very large unilamellar vesicles. But for the size, the giant mitochondria have similar characteristics to the native mitochondria. Their heme content, lipid content (Hochman et al., 1985), electron transfer (Hochman et al., 1982), and respiratory control (Maloff, 1978) are comparable to those found in normal rat liver mitochondria. The mitochondria (kept at 4 °C) are used within 10–14 h after preparation, and they are fairly stable in this time frame. NBDUQ-labeled inner mitochondrial membranes placed on a glass microscope slide for FRAP experiments are used within 15–45 min after the slide was prepared. Very large unilamellar vesicles (3–20 μm in diameter), prepared and fluorescently labeled as described under Experimental Procedures, were used within 10–15 min after being placed on a glass slide, since they tended to disintegrate. As the vesicles were somewhat mobile, gelatin-coated slides were used to retard the movement.

Incorporation of Fluorescent Probes into Membrane. Extent of incorporation of fluorescently labeled phospholipids is influenced by whether the fluorescent tag is located in the head or the acyl region (Struck & Pagano, 1980). Acyl-labeled phospholipids incorporate more easily than the head-group-labeled phospholipids. In the case of NBDUQ, sufficient incorporation to carry out FRAP measurement could be achieved in the presence of BSA (see Experimental Procedures), which seemed to act as a carrier protein for delivering NBDUQ into the mitochondrial membane. Final concentration of NBDUQ in the membrane was about 2–6 mol % with respect to lipid (≥2 times in excess of endogenous UQ). Head-group-labeled NBDPE was also incorporated better in the presence of BSA (to a level of ~1 mol %). Lower levels of NBDUQ could not be studied in mitochondria because of the relatively low fluorescence intensity of the NBDUQ compared to that of NBDPE. This difference could be seen in solvents where the fluroescence emission intensity of NBDPE was 2–4-fold greater than that of NBDUQ. It was also observed that the fluorescence emission intensity of NBDUQ was 3–4-fold greater in phospholipid vesicles than in mitochondrial membranes. This suggests that a significant portion of NBDUQ associated with the mitochondrial membranes may be in a quenched state. Such a quenched population would not contribute to the measurements of diffusion, fluorescence maxima, or fluorescence quenching.

Diffusion Coefficients of Fluorescence Probes in Membranes. The measured diffusion coefficients of NBDUQ in mitochondria and vesicles were 3.1 (±1.0) × 10^{-9} cm^2 s^{-1} and 1.1 (±0.2) × 10^{-8} cm^2 s^{-1}, respectively. The diffusion of NBDPE was similar to that of NBDUQ in both the systems (Table I). The recovery of the fluorescence after photobleaching of NBDUQ (Figure 5) was monophasic for more than 90% of the recovering species, in both the mitochondria and vesicles. The size of the vesicles was heterogeneous, diameters ranging from 3 to 20 μm. Diffusion coefficients seemed to show a slight dependence on the diameter of the vesicles (Rajarathnam, 1987), higher diffusion coefficients (1.5–2 times) being obtained at larger diameters. However, only the data obtained from vesicles of dimensions similar to those of the mitochondria were tabulated (Table I). Since the location of UQ in membranes may be concentration dependent, diffusion measurements were also performed as a function of concentration to see whether it had an effect on diffusion coefficients and the nature and extent of recovery. No discernible differences were observed (data not shown) when the experiments were performed over an 8-fold concentration range of 0.33-2.7 mol % NBDUQ/phospholipid in vesicles.

Comparison of Fluorescence Emission Maxima of Probes in Different Solvents and in Membranes. Table II shows the fluorescence emission maxima of NBDUQ, NBDPE, and IANBD measured in different organic solvents, in phospholipid vesicles, and in mitochondrial membranes. The emission maximum is often a sensitive indicator of the environment

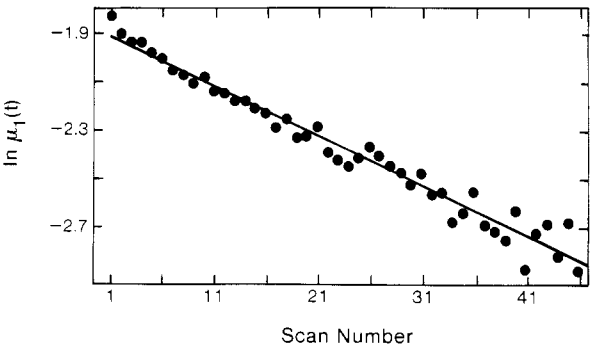

FIGURE 5: Fluorescence recovery profiles of NBDUQ in rat liver mitoplasts. Diffusion coefficients were measured and calculated with the edge bleach analysis technique described under Experimental Procedures. The sample shown was prepared by incubating 100 μL of mitoplasts (7.5 mg of protein/mL) with 5 μL of 0.1% BSA and 3 μL of NBDUQ (3 mg/mL) at room temperature for 10 min. The mitochondria were then swollen by addition of 3 volumes of distilled water on ice for 10 min. Scans were taken at 0.5-s intervals. The symbol $\mu_1(t)$ represents the fluorescence intensity gradient at time t and is defined as the normalized first moment of the unbleached fluorophore concentration distribution (Koppel et al., 1980).

Table II: Emission Maxima[a] of Fluorescent Probes in Organic Solvents, Phospholipid Vesicles, and Mitochondrial Membranes

solvent[b]	dielectric const (ε)[c]	emission max (nm)		
		NBDIA	NBDUQ	NBDPE
$CHCl_3$	4.9	516	516	519
CH_2Cl_2	8.9	520	521	519
1-propanol	20.3	531	530	530
acetone	20.7	530	529	528
EtOH	24.5	532	531	531
MeOH	32.7	535	533	533
H_2O	78.9	552	560	550
mitochondria[d]		536–538	527–529	535–537
vesicles		542–545	526–528	536–537

[a] Emission maxima were determined at an excitation of 468 nm in all cases. [b] Concentration of the fluorescent probes was 0.003 mg/mL in all the solvents. [c] Dielectric constants are from Marcus (1985). [d] Mitochondria and vesicles were prepared and labeled as described in the legend to Figure 5.

surrounding the fluorophore. The fluorescence emission maxima of the NBD fluorophore attached to these different molecules showed similar changes in response to different solvents. In general, there was a blue shift as the dielectric constant of the solvent decreased. However, the relationship between the emission maxima and the properties of the organic solvents is complex and could be dependent on parameters such as whether the solvent is protic or aprotic, its dipole moment, and the solubility of the compounds in that solvent. In apolar solvents, such as hexane and isooctane, the fluorescence emission was negligible and the spectrum very broad. It appeared likely that the NBDUQ was aggregating (insoluble) in these hydrocarbon solvents. When the fluorescent molecules were placed in an anisotropic environment, namely, in vesicles and mitochondria, significant differences were observed among the NBD-labeled molecules in their emission maxima. The emission maximum for NBDUQ was 525–527 nm in both the vesicles and mitochondria, significantly blue shifted compared to those of NBDPE (535–537 nm) and IANBD (542–544 nm), implying that NBDUQ is in a more hydrophobic environment.

Quenching of Fluorescent Probes in Membranes. Cu^{2+} is a water-soluble paramagnetic quenching agent that has been useful for locating the relative depth of fluorophores in a membrane by virtue of its ability to interact at the membrane surface and quench the fluorescence of the probes located on or near the surface (Thulborn & Sawyer, 1978). Thus Cu^{2+} was chosen to investigate the relative positions of NBDPE and NBDUQ in the mitochondrial membranes and vesicles. The data are presented in the form of Stern–Volmer plots where the quenching efficiency is related to the total quencher concentrations (Stern & Volmer, 1919):

$$I_0/I = 1 + k_q t_0[Q] \quad (1)$$

where I_0 and I are fluorescence intensities in the absence and presence of the quencher, [Q] is the quencher concentration, k_q is the bimolecular quenching constant, and t_0 is the lifetime of the fluorophore in the absence of the quencher. The quenching process in the membrane is complicated by the presence of both solution and membrane-associated forms of $CuSO_4$ (Blatt et al., 1986). Thus Stern-Volmer quenching constants ($k_{sv} = k_q t_0$) calculated from these data will be "apparent" values since the actual effective quencher concentration is not known. However, since the experiments were performed under nearly identical conditions for both NBDPE and NBDUQ in the vesicles and mitochondrial membranes, we can assume that the amount of bound and free Cu^{2+} will be the same for both of the probes.

In vesicles it is observed that quenching of head-group-labeled NBDPE was much higher than that of NBDUQ (Figure 6). The plots are nonlinear and show apparent saturation at higher Cu^{2+} levels. This behavior is expected in cases where total concentration rather than bound concentration is used on the abscissa and where the bound Cu^{2+} is assumed to be the most effective quencher (Thulborn & Sawyer, 1978). To calculate apparent Stern–Volmer quenching constants, slopes were calculated from the initial changes in fluorescence at low quencher concentrations, where it can be assumed that most of the quenching is occurring by a dynamic mechanism. In Table III it is seen that the apparent k_{sv} values in phospholipid vesicles are 5- to 6-fold higher for NBDPE than for NBDUQ. In mitochondria, the difference is even greater. In fact, there is essentially no quenching of NBDUQ, while quenching of NBDPE was more effective. The differences in k_{sv} values observed in phospholipid vesicles can be accounted for by a difference in lifetime between the two NBD probes, since the

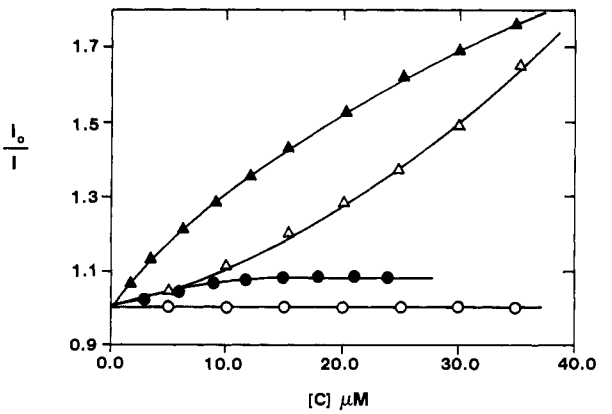

FIGURE 6: Stern–Volmer plots for quenching by $CuSO_4$ of NBDUQ (●, ○) and head-group-labeled NBDPE (▲, △) in giant mouse liver mitoplasts (open symbols) and phospholipid vesicles (closed symbols). Outer membrane depleted mitoplasts were labeled with the fluorescent probes as described in the legend to Figure 5 and diluted 3-fold for the fluorescence measurements. Vesicles containing the fluorescent probes (1:50 w/w) were prepared according to Mueller et al. (1983) and used as such for the fluorescence measurements (0.25 mg of phospholipid/mL). [C] represents the concentration of Cu^{2+}; I_0 is fluorescence intensity in the absence of Cu^{2+}; I is the fluorescence intensity in the presence of Cu^{2+}. Excitation was at 468 nm, and the emission was at 527 nm for NBDUQ and at 536 nm for NBDPE. Slit width was 5 nm for the excitation beam and 10–13 nm for the emission beam.

Table III: Quenching of NBDUQ and NBDPE by $CuSO_4$ in Mitochondria and Phospholipid Vesicles[a]

system/probe	k_{sv} (mM^{-1})	f_a (%)
mitochondria		
NBDUQ	0 (n = 6)	0
NBDPE	11 ± 2 (n = 7)	100
vesicles		
NBDUQ	7 ± 3 (n = 5)	15 ± 5
NBDPE	41 ± 4 (n = 4)	50 ± 9

[a] The apparent Stern–Volmer quenching constants (k_{sv}) were obtained from the initial slopes of data as shown in Figure 6. The fraction of the fluorophores quenched at infinite quencher concentration (f_a) was calculated from the y intercepts of data plotted according to the modified Stern–Volmer equation. Mitochondria and vesicles were prepared as detailed under Experimental Procedures. The values are given ± the standard deviation for the number of determinations indicated in parentheses.

average lifetimes differ by a factor of 5 (NBDPE, t_0 = 7.4 ns; NBDUQ, t_0 = 1.5 ns).[2] However, the total lack of quenching in the mitochondria cannot be accounted for in this way; the lifetime differences will only affect the apparent quenching constants, not the extent of quenching, when both values are in a range where quenching is effective.

The extent of quenching in a membrane (Omann & Glaser, 1984) can be obtained from the modified Stern–Volmer equation (Lehrer, 1971):

$$\frac{I_0}{I_0 - I} = \frac{1}{[Q] f_a k} + \frac{1}{f_a} \quad (2)$$

where f_a is the fraction of the fluorophore accessible to the quencher. A plot of $I_0/(I_0 - I)$ vs $1/[Q]$ yields a straight line (data not shown), and the intercept gives the extent of

[2] Lifetime measurements on NBDUQ were made by J. Vanderkooi, University of Pennsylvania, Philadelphia. The values given are the averages of biphasic exponential decay curves where the two phases had approximately equal intensities. In phospholipid vesicles, t_1 = 0.6 ns, t_2 = 2.4 ns, and average t_0 = 1.5 ns; in ethanol, t_1 = 0.6 ns and t_2 = 1.4 ns. These are compared to the values reported by Arvinte et al. (1986) for NBDPE in phospholipid vesicles (also biphasic but unequal intensities): t_1 = 2.4 ns, t_2 = 8.5 ns, and weighted average t_0 = 7.4 ns.

quenching at infinite concentrations of the quencher. The intercepts indicate that about 40–50% of NBDPE is quenched in the vesicles and 100% is quenched in the mitochondria (Table III). For NBDUQ, about 15% can be quenched in the vesicles, but none is quenched in the mitochondria. In the vesicles, which were formed after the probe and phospholipid were mixed, about 50% of the probe would be expected to be located in the outer leaflet of the bilayer and thus accessible to the quencher (as observed for NBDPE). In mitochondria, the probe is added exogenously and would be located only in the outer leaflet since the flip-flop process for phospholipids is very slow in the natural membranes (hence, 100% accessible). It is clear from the overall quenching data that NBDUQ is much less accessible to Cu^{2+} than is NBDPE in both the membrane systems, indicating that it partitions into a more hydrophobic environment.

Effect of Fluorescent Probes on Electron Transfer in Mitoplasts. The effect of NBDPE and NBDUQ on uncoupled electron transfer from succinate to oxygen was measured in mitoplasts under similar loading conditions to those used in the fluorescence studies. No effect on the rates of electron transfer was observed, indicating that the fluorescent probes are not disrupting the native membrane. Since the head group of UQ is modified, it would not be expected to bind at the Q-binding sites involved in electron transfer and hence would not be able to compete with the endogenous UQ.

Discussion

Synthesis of NBDCOQ. We have synthesized a fluorescent derivative of ubiquinone (NBDUQ) where the fluorescent moiety is attached to the head group. NBD was chosen as the fluorescent tag as it is relatively small and hydrophobic, has an absorbance maximum in the visible region with a fairly high extinction coefficient, and is commercially available as an alkyl halide (IANBD). Reduced UQ was reacted with IANBD using DMAP as a catalyst in THF to obtain NBDUQ (yield = 5%). DMAP is a widely used, highly active acylation catalyst (Hofle et al., 1978) and has been shown to be far superior to pyridine, but it is not generally used as an alkylation catalyst. In our case, it turned out to be very effective since DMAP is a better leaving group than iodide. The reduced UQ could act as an effective nucleophile to displace DMAP and form a phenoxy bond to give NBDUQ. Attempts to activate reduced UQ by converting into a phenoxide by use of potassium *tert*-butoxide as a base resulted in the product but also gave other products, and the reaction was not very reproducible as it was extremely sensitive to humidity. DMAP is a milder catalyst, and NBDUQ is obtained in a more reproducible manner. The general usefulness of the procedure as a method for modifying the quinone head group is illustrated by the successful reaction with another fluorescent probe, Thiolyte. The properties of this UQ derivative have not yet been extensively investigated.

The reaction conditions were mild, and the purification of the product could be achieved by preparative TLC in one step. The product was reasonably stable when kept frozen in dried form. The excitation and emission properties were similar to those of NBDPE though extinction coefficients were lower. Fluorescence lifetime of NBDUQ was shorter (by a factor of $\sim$5) but was in a reasonable range for performing quenching experiments.

Location of NBDUQ. The fluorescence quenching and emission studies of NBDUQ in both vesicles and mitochondria reported in this paper are consistent with a location for NBDUQ deep in the membrane. The emission maximum of NBDUQ is blue shifted at least by 10 nm compared to that of a head-group-labeled phospholipid in mitochondria and vesicles. In mitochondria, no quenching of NBDUQ by $CuSO_4$ was observed while NBDPE was totally quenched, indicating that the fluorescent head group of NBDUQ was not accessible to the surface of the membrane. However, these findings do not rule out the existence of a small population (<5%) of NBDUQ located near the membrane surface, which our methods would not be sensitive enough to distinguish. In phospholipid vesicles there appears to be a measurable fraction ($\sim$10–15%) of NBDUQ accessible to $CuSO_4$. Attempts were made to estimate the depth of the NBDUQ in the membrane by comparing its quenching behavior with that of NBDPE labeled in the 6- and 12-positions of the alkyl chain. However, these studies were not informative because of the tendency of the alkyl chains with the probe attached to loop back to the membrane surface (Chattopadhyay & London, 1987).

A variety of physical techniques such as NMR (Kingsley & Feigenson, 1981; Stidham et al., 1984; Ulrich et al., 1985; Michaelis & Moore, 1985; Ondarroa & Quinn, 1986; Cornell et al., 1987), differential scanning calorimetry (Katsikas & Quinn, 1982; Aranda & Gomez-Fernandez, 1985), infrared (Aranda et al., 1986), absorption (Degli Esposti et al., 1981), and fluorescence measurements (Katsikas & Quinn, 1983; Chatelier & Sawyer, 1985; Aranda & Gomez-Fernandez, 1986) have been applied to measure the location of ubiquinone. Most of the studies suggest that ubiquinone is located in a hydrophobic region of the membrane, but there is disagreement regarding the orientation, the state of aggregation, and the existence of more than one population in the membrane. The most widely accepted model suggests that the isoprene tail and the head group lie in the midplane of the bilayer (Quinn & Esfahani, 1980; Ulrich et al., 1985; Cornell et al., 1987). Another model places the isoprene tail in the midplane but the head group in the phospholipid leaflet, where it can have access to the membrane surface (Mitchell, 1976; Stidham et al., 1984). A model in which UQ can act as a micelle spanning the bilayer has also been proposed (Ondarroa & Quinn, 1986).

MacDonald (1987) discusses partitioning of hydrophobic molecules into membranes and proposes that molecules having the same surface pressure as a phospholipid can freely intercalate with the phospholipids, but if the surface pressure is significantly less, intercalation is not favorable, and most of the molecules would partition deeper into the membrane. The surface pressure of UQ is fairly low (12 dyn/cm) compared to the surface pressure of a phospholipid (35 dyn/cm) (Quinn & Esfahani, 1980), and hence, it is likely that a major portion of UQ partitions deep into the membrane.

Location of UQ in the native mitochondria is complicated by the fact that the inner membrane has a high protein concentration, including Q-binding proteins that may affect its orientation and location (Yu & Yu, 1980; King & Suzuki, 1984; Yu et al., 1985). Since the redox reactions of UQ involve protons, communication with an aqueous environment would appear necessary. Rich (1984) proposes that UQ and UQH_2 are in such an environment (hydrophobic) that they are essentially unreactive until they collide with an appropriate site on a donor or acceptor protein which then can facilitate their access to the aqueous phase, thus ensuring the biological specificity of the reaction route.

Diffusion of NBDUQ in Vesicles and Mitochondria. The diffusion coefficients measured for NBDUQ and NBDPE are very similar, and both show $\sim$4-fold higher diffusion rates in artificial phospholipid vesicles compared to mitochondrial membranes. Even though the loading of mitochondria with NBDUQ (2–6 mol %) was somewhat in excess of physiological

levels (1–2 mol %), studies with the phospholipid vesicles over an 8-fold concentration range (0.33–2.7 mol %) showed no change in diffusion coefficients, indicating this is not likely to be a major source of error. The difference in mobility of NBDUQ in phospholipid vesicles and mitochondrial membranes is also consistent with diffusion measurements in a number of systems [see review by McCloskey and Poo (1986)] that show slower diffusion rates in protein-containing compared to pure phospholipid membranes. Nevertheless, the similarity in diffusion rates between NBDPE and NBDUQ in both systems led to the concern that the NBD moiety attached to the quinone head group might cause it to be pulled nearer to the membrane surface (Chattopadhyay & London, 1987), resulting in a phospholipid-like behavior. However, the fluorescence emission and fluorescence quenching studies reported here indicate that NBDUQ is in a different location than a phospholipid, a more hydrophobic environment not accessible to the membrane surface. It appears that any tendency for the NBD moiety to draw the quinone moiety to the membrane surface is counteracted by the hydrophobic and rigid character of the isoprene side chain. Nevertheless, the similarity between NBDUQ and NBDPE mobilities could be interpreted to indicate that the diffusion of UQ is limited by the diffusion of the phospholipid. If UQ diffuses at the same rate as a phospholipid, it would imply that UQ does not occupy a completely independent phase. The free-volume theory postulated by Cohen and Turnbull (1959) predicts that a solute can diffuse no faster than its solvent since a diffusive step of the solute is only completed when a solvent molecule moves into the void left by the solute (Vaz et al., 1984). A model consistent with these data [and similar to that proposed by Stidham et al. (1984)] would have the major part of the isoprene chain in the midplane while the head group penetrates to some degree among the phospholipid acyl chains but not into the hydrophilic region. Alternatively, the majority of the quinone molecules may occupy a separate phase in the midplane of the bilayer but still exhibit diffusion rates comparable to those of a phospholipid. It is an interesting possibility that the modified UQ may behave more like ubiquinol, which might be expected to have higher surface pressure than ubiquinone and thus penetrate to a somewhat greater extent into the phospholipid leaflet. In any case, our data do not support the hypothesis that there is a highly mobile ($D = 10^{-6}$ cm^2 s^{-1}) population of ubiquinone in the membrane (Millner & Barber, 1984).

The fluorescence quenching studies of Lenaz and colleagues, which give a diffusion coefficient of 10^{-6}–10^{-7} cm^2 s^{-1}, measure only short-range diffusion while FRAP measures long-range diffusion. It is argued by Lenaz and co-workers that short-range diffusion is of greater physiological significance (Fato et al., 1986). However, long-range diffusion rates in artificial phospholipid vesicles should be similar to short-range diffusion rates in mitochondrial membranes, as there are no proteins to retard the movement (Vaz et al., 1984). Nevertheless, the diffusion value of 1×10^{-6} cm^2 s^{-1} is still 2 orders of magnitude higher than what we observe by FRAP in artificial vesicles. With regard to this discrepancy, Blackwell et al. (1987) using the quenching data of Lenaz's group and an alternative empirical method for calculating diffusion coefficients obtained a diffusion coefficient for ubiquinone on the order of 10^{-7} cm^2 s^{-1}. Lenaz and colleagues concur with the lower estimate (Lenaz et al., 1988). Thus, it is reasonable to conclude that the value of 10^{-6} cm^2 s^{-1} originally calculated by Fato et al. (1986) is an overestimate by at least 1 order of magnitude, but that still leaves an order of magnitude difference between the FRAP analysis and the collisional quenching analysis. Our results indicate that this discrepancy cannot be accounted for by the nature of the isoprene chain alone and support the conclusion that diffusion of UQ is not extremely fast.

Whether UQ mobility is rate limiting under physiological conditions in mitochondria remains to be determined. As yet there are few data regarding the organization (or lack thereof) of electron transfer complexes within the mitochondrial inner membrane. The available evidence, biochemical (Werner & Neupert, 1972; Yu & Yu, 1980; Gwak et al., 1986; Møller, 1988), biophysical (Silverstein & Rottenberg, 1987), and ultrastructural (Srere, 1982; Sjostrand, 1983; Staehelin & Arntzen, 1983), strongly indicates a nonrandom distribution of proteins in energy-transducing membranes. Such nonrandom organization may increase or decrease the path length of UQ diffusion. Given the additional complexity of protein-bound forms of UQ (Yu & Yu, 1980; King & Suzuki, 1984; Yu et al., 1985), it is reasonable to suggest [consistent with the continuum model of Rich (1984)] at least three different pools of UQ: (1) randomly distributed, with long-range diffusion rates of 10^{-9} cm^2 s^{-1}; (2) localized domains, with short-range diffusion rates of 10^{-8} cm^2 s^{-1}; (3) protein bound, with diffusion rates of 10^{-10} cm^2 s^{-1}. Determining the size and functional significance of these pools under various physiological conditions will require further investigation.

Acknowledgments

We are very grateful to Dr. Jane Vanderkooi (University of Pennsylvania, Philadelphia) for fluorescence lifetime measurements on NBDUQ and for valuable discussions. We thank Brian Musselman and the Michigan State University Mass Spectrometry Facility (NIH Grant RR-00480) for mass spectral analysis of the structure of NBDUQ.

Registry No. UQ, 303-98-0; IANBD, 67013-48-3; NBDUQ, 119110-36-0; NBDPE, 99684-86-3; α-tocopherol, 59-02-9.

References

Aranda, F. J., & Gomez-Fernandez, J. C. (1985) *Biochim. Biophys. Acta 820*, 19–26.

Aranda, F. J., & Gomez-Fernandez, J. C. (1986) *Biochem. Int. 12*, 137–143.

Aranda, F. J., Villalain, J., & Gomez-Fernandez, J. C. (1986) *Biochim. Biophys. Acta 861*, 25–32.

Arvinte, T., Cudd, A., & Hildenbrand, K. (1986) *Biochim. Biophys. Acta 860*, 215–228.

Badley, R. A., Martin, W. S., & Schneider, H. (1973) *Biochemistry 12*, 268–275.

Blackwell, M. F., Gounaris, K., Zara, S. J., & Barber, J. (1987) *Biophys. J. 51*, 735–744.

Blatt, E., Chatelier, R. C., & Sawyer, W. H. (1986) *Biophys. J. 50*, 349–356.

Bowman, C. L., & Tedeshi, H. (1983) *Biochim. Biophys. Acta 731*, 261–266.

Capaldi, R. A. (1982) *Biochim. Biophys. Acta 694*, 291–306.

Chatelier, R. C., & Sawyer, W. H. (1985) *Eur. Biophys. J. 11*, 179–185.

Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39–45.

Cohen, M. H., & Turnbull, D. (1959) *J. Chem. Phys. 31*, 1164–1169.

Cornell, B. A., Keniry, M., Post, A., Robertson, R., Weir, L., & Westerman, P. (1987) *Biochemistry 26*, 7702–7707.

Crane, F. L. (1977) *Annu. Rev. Biochem. 46*, 439–469.

Degli Esposti, M., Bertoli, E., Parenti-Castelli, G., Fato, R., Mascarello, S., & Lenaz, G. (1981) *Arch. Biochem. Biophys. 210*, 21–32.

Fahey, P. F., & Webb, W. W. (1978) *Biochemistry 17*, 3046–3053.
Fato, R., Battino, M., Castelli, G. P., & Lenaz, G. (1985) *FEBS Lett. 179*, 238–242.
Fato, R., Battino, M., Degli Esposti, M., Castelli, G. P., & Lenaz, G. (1986) *Biochemistry 25*, 3378–3390.
Green, D. E. (1962) *Comp. Biochem. Physiol. 4*, 81–122.
Gupte, S. S., Wu, E. S., Hoechli, L., Hoechli, M., Jacobson, K., Sowers, A. E., & Hackenbrock, C. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2606–2610.
Gwak, S. H., Yu, L., & Yu, C. A. (1986) *Biochemistry 25*, 7675–7682.
Hackenbrock, C. R., Chazotte, B., & Gupte, S. S. (1986a) *J. Bioenerg. Biomembr. 18*, 331–368.
Hackenbrock, C. R., Chazotte, B., & Gupte, S. S. (1986b) in *Biomedical and Clinical Aspects of Coenzymes Q* (Folkers, K., & Yamamura, Y., Eds.) Vol. 5, pp 43–55, Elsevier, Amsterdam.
Hochman, J., Schindler, M., Lee, J. G., & Ferguson-Miller, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6866–6870.
Hochman, J., Ferguson-Miller, S., & Schindler, M. (1985) *Biochemistry 24*, 2509–2516.
Hofle, G., Steglich, W., & Vorbruggen, H. (1978) *Angew. Chem., Int. Ed. Engl. 17*, 569–583.
Katayama, et al. (1980) *Biochem. Biophys. Res. Commun. 95*, 971.
Katsikas, H., & Quinn, P. J. (1982) *Biochim. Biophys. Acta 689*, 363–369.
Katsikas, H., & Quinn, P. J. (1983) *Eur. J. Biochem. 131*, 607–612.
King, T. E., & Suzuki, H. (1984) in *Biomedical and Clinical Aspects of Coenzyme Q* (Folkers, K., & Yamamura, Y., Eds.) Vol. 4, pp 43–55, Elsevier, Amsterdam.
Kingsley, P. B., & Feigenson, G. (1981) *Biochim. Biophys. Acta 635*, 602–618.
Koppel, D. E. (1979) *Biophys. J. 28*, 281–292.
Koppel, D. E., Sheetz, M. P., & Schindler, M. (1980) *Biophys. J. 30*, 187–192.
Lakowicz, J. R., & Hogen, D. (1980) *Chem. Phys. Lipids 26*, 1–40.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.
Lenaz, G., Battino, M., Castelluccio, C. Fato, R., & Parenti-Castelli, G. (1988) *EBEC Rep. 5*, 44.
MacDonald, R. C. (1987) *Biophys. J. 51*, Abstr. M-319.
Maloff, B. L., Scordiles, S. P., Reynolds, C., & Tedeschi, H. (1978) *J. Cell Biol. 78*, 199–213.
Marcus, Y. (1985) *Ion Solvation*, pp 136–137, Wiley, New York.
McCloskey, M. A., & Poo, M. (1986) *J. Cell Biol. 102*, 88–96.
Michaelis, L., & Moore, M. J. (1985) *Biochim. Biophys. Acta 821*, 121–129.
Millner, P. A., & Barber, J. (1984) *FEBS Lett. 169*, 1–6.
Mitchell, P. (1976) *J. Theor. Biol. 62*, 327–367.
Møller, I. M. (1988) *Physiol. Plant. 73*, 153–157.
Mueller, P., Chien, T. F., & Rudy, B. (1983) *Biophys. J. 44*, 375–381.
Omann, G. M., & Glazer, M. (1984) *Biochemistry 23*, 4962–4969.
Ondarroa, M., & Quinn, P. J. (1986) *Eur. J. Biochem. 155*, 353–361.
Quinn, P. J., & Esfahani, M. A. (1980) *Biochem. J. 185*, 715–722.
Rajarathnam, K. (1987) M.S. Thesis, Michigan State University.
Schneider, H., Lemasters, J. J., & Hackenbrock, C. R. (1982) *J. Biol. Chem. 257*, 10789–10793.
Silverstein, T. P., & Rottenberg, H. (1987) *Biophys. J. 51*, Abstr. W-192.
Sjostrand, F. S. (1983) in *Membrane Fluidity in Biology* (Aloia, R. C., Ed.) Vol. 1, pp 83–142, Academic Press, New York.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. T., Gartner, F. H., Provenzano, M. D., Fujimato, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Srere, P. A. (1982) *Trends Biochem. Sci. 82*, 375–378.
Staehelin, L. A., & Arntzen, C. J. (1983) *J. Cell Biol. 97*, 1327–1337.
Stern, O., & Volmer, M. (1919) *Phys. Z. 20*, 183–189.
Stidham, M. A., McIntosh, T. J., & Siedow, J. N. (1984) *Biochim. Biophys. Acta 767*, 423–431.
Struck, D. K., & Pagano, R. E. (1980) *J. Biol. Chem. 255*, 5404–5410.
Thulborn, K. R., & Sawyer, W. H. (1978) *Biochim. Biophys. Acta 511*, 125–140.
Trumpower, B. L. (1981) *J. Bioenerg. Biomembr. 13*, 1–24.
Ulrich, E. L., Girvin, M. E., Cramer, W. A., & Markley, J. L. (1985) *Biochemistry 24*, 2501–2508.
Vaz, W. L. C., Goodsaid-Zalduondo, F., & Jacobson, K. (1984) *FEBS Lett. 174*, 199–207.
Werner, S., & Neupert, W. (1972) *Eur. J. Biochem. 25*, 379–396.
Yu, C. A., & Yu, L. (1980) *Biochim. Biophys. Acta 591*, 409–420.
Yu, L., Yang, F. D., & Yu, C. A. (1985) *J. Biol. Chem. 260*, 963–973.